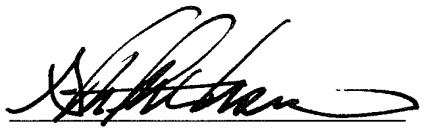
DECLARATION

I, Shinichi Usui, a Japanese Patent Attorney registered No. 9694, of Okabe International Patent Office at No. 602, Fuji Bldg., 2-3, Marunouchi 3-chome, Chiyoda-ku, Tokyo, Japan, hereby declare that I have a thorough knowledge of Japanese and English languages, and that the attached pages contain a correct translation into English of the priority documents of Japanese Patent Application No. 2003-418560 filed on December 16, 2003 in the name of CANON KABUSHIKI KAISHA.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

signed this 15 th day of September, 2010



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JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of the following application as filed with this Office.

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[List of Filed Materials]

[Material]	Claims	1
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2003-418560

[Name of the Document] CLAIMS

[Claim 1]

A cell culture substrate having at least one area for culturing a cell on a substrate, characterized in that the culturing area comprises an area for holding a biologically active substance having a biological activity to the cell and an area for immobilizing a biologically active substance having a biological activity to the cell.

[Claim 2]

The cell culture substrate according to claim 1, wherein a plurality of biologically active substances are held or immobilized in at least either of the holding area and the immobilizing area in each culture area.

[Claim 3]

The cell culture substrate according to claim 1 or 2, wherein the biologically active substance in the holding area is held in such a manner that it is released in a culture liquid when coming in contact with the holding area.

[Claim 4]

The cell culture substrate according to any one of claims 1 to 3, wherein at least either of the holding area and the immobilizing area in the culture area is provided in plural units.

[Claim 5]

The cell culture substrate according to any one of claims 1 to 4, wherein the holding area and the immobilizing area include areas between which the kind of the biologically active substance or the combination of plural biologically active substances is different, and one or more combinations are included.

[Claim 6]

The cell culture substrate according to any one of claims 1 to 5, wherein the holding areas and the immobilizing areas include at least a combination of the areas different in a density of the biologically active substance.

[Claim 7]

The cell culture substrate according to any one of claims 1 to 6, wherein the culture area is formed in a recess formed on a surface of the substrate.

[Claim 8]

The cell culture substrate according to any one of claims 1 to 6, wherein the culture area is surrounded by a wall-shaped structure.

[Claim 9]

The cell culture substrate according to any one of claims 1 to 8, wherein at least either of the holding area and the immobilizing area includes an area in which a biologically active substance is held

or immobilized across a supporting layer provided on a surface of the substrate.

[Claim 10]

The cell culture substrate according to any one of claims 1 to 9, wherein the holding area is provided at a predetermined height from a lower end of the culture area.

[Claim 11]

The cell culture substrate according to claim 10, wherein a culture area includes two or more holding areas provided in positions different in distances from a lower end of the culture area.

[Claim 12]

The cell culture substrate according to any one of claims 1 to 11, characterized in that the biologically active substance can be control-released or a biologically active substance having a control-release property can be liberated from the holding area.

[Claim 13]

The cell culture substrate according to any one of claims 1 to 12, wherein the culture area is provided in a recess, of which at least one of walls is inclined from a bottom portion to an upper portion, and an aperture of the recess has an area wider than a bottom area of the recess.

[Claim 14]

A method for producing a cell culture substrate according to any one of claims 1 to 13, characterized in that liquid discharge means is utilized for providing a biologically active substance to at least one of the holding area and the immobilizing area.

[Claim 15]

The method according to claim 14, wherein the liquid discharge means is discharge means by a thermal ink jet method.

[Claim 16]

The method according to claim 14, wherein the liquid discharge means is discharge means by a piezo ink jet method.

[Claim 17]

The method according to any one of claims 14 to 16, further comprising a step of carrying out fixation of the biologically active substance by applying an immobilizing energy from the exterior.

[Claim 18]

A method for screening a cell utilizing a cell culture substrate according to any of claims 1 to 13, the method comprising the steps of:

filling the culture area with a culture liquid and culturing cells in a state where a biologically active substance immobilized in an immobilizing area of the culture area is in contact with the culture liquid; and

contacting the culture liquid with the holding area thereby liberating a biologically active substance present in the holding area into the culture liquid.

[Claim 19]

The method according to claim 18, further comprising the step of replenishing the culture liquid with a substance necessary for screening a cell.

[Claim 20]

The method according to claim 18 or 19, further comprising the step of observing a shape change of cells.

[Claim 21]

The method according to claim 20, wherein cells are stained for evaluation.

[Claim 22]

The method according to any one of claims 18 to 21, further comprising the step of carrying out a quantitative determination of a substance synthesized in the cells.

[Claim 23]

The method according to any one of claims 18 to 21, further comprising the step of carrying out a quantitative determination of a substance incorporated in the cells.

[Claim 24]

The method according to claim 22 or 23, further comprising a step of carrying out a quantitative determination of the substance by at least one of a radiation dose measurement, a fluorescence amount measurement, a light emission amount measurement and an optical absorbance measurement.

[Name of the Document] Specification

[Title of the Invention] Cell Culturing Substrate, Method of Producing the Same and Cell Screening Method Using the Same

[Technical Field]

[0001]

The present invention relates to a substrate for culturing cells for identifying a biologically active substance having a biological activity to cells, a producing method thereof and a cell screening method utilizing the same.

[Background Art]

[0002]

In recent years, studies of culturing animal or plant cells under various conditions and studies of products of certain cell cultures have been actively carried out. Particularly, studies on production of substances, of which artificial synthesis is impossible or very difficult, utilizing certain cell activities are carried out in various fields. Also studies are carried out to identify substances that affect cellular growth and differentiation so as to obtain proliferation or differentiation of certain cells according to the purpose. Also with the rapid progress in cell engineering and medical engineering, minute biosensors, artificial organs, neurocomputers and the like using cells are attracting attentions

and actively studied. In order to utilize cells in vitro as explained above, it is essential to dispose cells to control their proliferation, differentiation and substance production in a desired manner. However, the mechanisms of cell disposition, cell proliferation and differentiation and substance production have not been sufficiently clarified, so that the cell culture under controlled conditions is extremely difficult and impeding researches utilizing cells as mentioned above.

[0003]

Also tailor-made therapy considering personal difference in the drug sensitivity, of which concept has recently been widely recognized, is strongly desired, but the influence of biologically active substances has been investigated only on the function of respective substances, mainly because of technical reasons, and there has not been established an effective method for easily investigating effects of plural drugs at the same time, or required doses thereof or combined effects thereof.

[0004]

For controlling cell disposition, USP No. 5,108,926 describes formation a pattern on a substrate by applying a cell-adhering protein using an ink jet printer and cell culture thereon. This method allows to culture cells on the formed pattern

of the cell-adhering protein, but not to control the proliferation, differentiation and substance production of the cells or to achieve screening of cells. Also "Protein, Nucleic acid and Enzyme", vol. 45, 727-734(2000) describes fixation of a cell growth factor that influences the cell proliferation and differentiation on a substrate using photolithography, thereby investigating its influence on the cell proliferation and differentiation. However, the substrate on which the cell growth factor is immobilized is not used for screening of cells, and the photolithography has problems that a rare biological substance is wasted and the production process is complicated requiring repetition of exposure and development steps.

[0005]

Japanese translation of PCT international application No. 2000-512009 proposes a method for screening of cells by immobilizing onto a substrate a substance that affects cell adhesion. In this method, reactive functional groups provided on the substrate and the cell-adhering substance are bonded through a divalent crosslinking reagent. Photolithographic technology is utilized in bonding the reactive functional group and the cell-adhering substance, which has problems, in addition to the aforementioned problems, that when plural cell-adhering substances

are immobilized, it is extremely difficult to avoid a situation where an already immobilized substance and a substance to be newly immobilized are bonded by the divalent crosslinking reagent in undesired positions, that is, it is extremely difficult to arrange cell-adhering substances in desired positions. Also the proposed method is not to fix a substance influencing the cell proliferation, differentiation and substance production. That method is to screen cells by immobilizing cells in individual wells through an adhering substance, culturing the cells in a culture medium and detecting a certain substance produced by the cells. Thus it is not intended for screening a substance which influences at least one of adhesive property, proliferation, differentiation, survival, maintenance of an undifferentiated state, death and material production of cells, as intended in the present invention.

[0006]

Also Japanese Patent Application Laid-open No. 2002-355025 discloses a method of forming a cell screening substrate characterized in immobilizing plural cell screening substances by using liquid discharge means in desired areas of a base, thereby providing the areas with different screening functions. In this invention, since the substances for cell screening are all immobilized to the

screening substrate in a cell culture liquid, the cell screening substance cannot be taken into the cells in many cases. Therefore, this invention is effective, except the case of screening cells where at least one of proliferation, differentiation, survival, maintenance of an undifferentiated state, death and substance production is affected when the cell screening substance is taken into the cells.

[0007]

Also Japanese Patent Application Laid-open No. 2002-328124 discloses screening of a higher order combination of biological active substances, but this is to evaluate an effect of a function of biological active substances previously provided to a substrate, so it cannot be used for screening of effects of biological active substances that affect the living body in a state immobilized on an extra-cellular substrate in the living body, or an effect induced by successive additions of biological active substances.

Also Japanese Patent Application Laid-open No. 2003-33177 proposes a simple assay of chemical substances such as various drugs or toxic substances, preparing a cell array divided into plural areas and providing a biologically active substance to each area to carry out simultaneous screening of plural samples. In such a method, however, each biologically active substance is provided to the

cultured cells by using a dispensing means. Thus there is a danger of contamination in the dispensing step and it requires a specific apparatus for dispensing the biologically active substances, far from convenient use.

[Patent Document 1] USP No. 5,108,926

[Patent Document 2] PCT International Application No. 2000-512009

[Patent Document 3] Japanese Patent Application Laid-open No. 2002-355025

[Patent Document 4] Japanese Patent Application Laid-open No. 2002-328124

[Patent Document 5] Japanese Patent Application Laid-open No. 2003-33177

[Non Patent Document 1] "Protein, Nucleic Acid and Enzyme", vol. 45, 727-734(2000)

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

[0008]

In consideration of the foregoing, the present invention aims to provide a cell culture kit which can solve the technical problems in the aforementioned prior techniques and enables simultaneous evaluation of the effects of plural biologically active substances in a immobilized or dissolved state through simple steps, as well as a producing method of such a kit and a screening method

utilizing the same, thereby providing a basic technology for further advance in cell engineering and for various cell-utilizing devices. Another object of the present invention is to provide a screening method utilizing such a cell culture kit, for screening a substance which influences at least one of all the biologically active substances having an activity to a cell. Still another object of the invention is to provide a method of screening a biologically active substance and/or condition utilizing cells.

[Means for Solving the Problems]

[0009]

The present invention includes following aspects:

(1) A cell culture substrate having at least one area for culturing a cell on a substrate, characterized in that the culturing area comprises an area for holding a biologically active substance having a biological activity to the cell and an area for immobilizing a biologically active substance having a biological activity to the cell.

(2) The cell culture substrate according to the above item (1), wherein a plurality of biologically active substances are held or immobilized in at least either of the holding area and the immobilizing area in each culture area.

(3) The cell culture substrate according to the above item (1) or (2), wherein the biologically active substance in the holding area is held in such a manner that it is released in a culture liquid when coming in contact with the holding area.

(4) The cell culture substrate according to any one of the above items (1) to (3), wherein at least either of the holding area and the immobilizing area in the culture area is provided in plural units.

(5) The cell culture substrate according to any one of the above items (1) to (4), wherein the holding area and the immobilizing area include areas between which the kind of the biologically active substance or the combination of plural biologically active substances is different, and one or more combinations are included.

(6) The cell culture substrate according to any one of the above items (1) to (5), wherein the holding areas and the immobilizing areas include at least a combination of the areas different in a density of the biologically active substance.

(7) The cell culture substrate according to any one of the above items (1) to (6), wherein the culture area is formed in a recess formed on a surface of the substrate.

(8) The cell culture substrate according to any one of the above items (1) to (6), wherein the

culture area is surrounded by a wall-shaped structure.

(9) The cell culture substrate according to any one of the above items (1) to (8), wherein at least either of the holding area and the immobilizing area includes an area in which a biologically active substance is held or immobilized across a supporting layer provided on a surface of the substrate.

(10) The cell culture substrate according to any one of the above items (1) to (9), wherein the holding area is provided at a predetermined height from a lower end of the culture area.

(11) The cell culture substrate according to the above item (10), wherein a culture area includes two or more holding areas provided in positions different in distances from a lower end of the culture area.

(12) The cell culture substrate according to any one of the above items (1) to (11), characterized in that the biologically active substance can be control-released or a biologically active substance having a control-release property can be liberated from the holding area.

(13) The cell culture substrate according to any one of the above items (1) to (12), wherein the culture area is provided in a recess, of which at least one of walls is inclined from a bottom portion to an upper portion, and an aperture of the recess has an area wider than a bottom area of the recess.

(14) A method for producing a cell culture substrate according to any one of the above items (1) to (13), characterized in that liquid discharge means is utilized for providing a biologically active substance to at least one of the holding area and the immobilizing area.

(15) The method according to the above item (14), wherein the liquid discharge means is discharge means by a thermal ink jet method.

(16) The method according to the above item (14), wherein the liquid discharge means is discharge means by a piezo ink jet method.

(17) The method according to any one of the above items (14) to (16), further comprising a step of carrying out fixation of the biologically active substance by applying an immobilizing energy from the exterior.

(18) A method for screening a cell utilizing a cell culture substrate according to any one of the above items (1) to (13), the method comprising the steps of:

filling the culture area with a culture liquid and culturing cells in a state where a biologically active substance immobilized in an immobilizing area of the culture area is in contact with the culture liquid; and

contacting the culture liquid with the holding

area thereby liberating a biologically active substance present in the holding area into the culture liquid.

(19) The method according to the above item (18), further comprising the step of replenishing the culture liquid with a substance necessary for screening a cell.

(20) The method according to the above item (18) or (19), further comprising the step of observing a shape change of cells.

(21) The method according to the above item (20), wherein cells are stained for evaluation.

(22) The method according to any one of the above items (18) to (21), further comprising the step of carrying out a quantitative determination of a substance synthesized in the cells.

(23) The method according to any one of the above items (18) to (21), further comprising the step of carrying out a quantitative determination of a substance incorporated in the cells.

(24) The method according to the above item (22) or (23), further comprising a step of carrying out a quantitative determination of the substance by at least one of a radiation dose measurement, a fluorescence amount measurement, a light emission amount measurement and an optical absorbance measurement.

[Effect of the Invention]

[0010]

The screening method of the invention enables identification of a factor required for cell proliferation, differentiation, survival, maintenance of undifferentiated state, death or production of substances, which allows determination of an effective cell-culturing method. Also according to the present invention, screening of substances can be used not only in their solid phase but also in their liquid phase, that is, screening is carried out under conditions closer to *in vivo* conditions using combinations of biologically active substances in their immobilized state or dissolved state. The present invention also allows investigation of difference in the effect in a solid phase and in a liquid phase, and evaluation of individual sensitivity to a drug or an endocrine perturbing substance, so-called environmental hormone. It is also possible, based on the result of such evaluation, to determine a tailor made therapeutic method for various diseases. It is furthermore possible to screen useful substances having biological activities to cells using cells.

[0011]

The cell culture substrate of the invention can be produced employing an ink jet method as the liquid

discharge means, enabling simultaneous action of plural substances on the cells at various concentrations by controlling the number of liquid droplets. Also the culture substrate of the invention allows precise re-solubilization of plural cell screening substances in a cell culture liquid, enabling exact evaluation of an effect of a system comprised of plural biologically active substances to the cell proliferation, differentiation and survival, which has been difficult to evaluate in the prior technology. Also a successive addition of biologically active substances or change in combination is easy. Therefore, screening for the effect of such conditions can be performed easily and effectively. Furthermore, the cell culture substrate of the invention can be easily produced in a large scale, and stored stably and used whenever desired as a screening substrate.

[Best Mode for Carrying Out the Invention]

[0012]

A best mode of the present invention is a cell culture substrate 1 having a biologically active substance, formed by holding (i.e., temporarily immobilizing) on a substrate 1 a biologically active substance that affects at least one function selected from adhesion, proliferation, differentiation, survival, maintenance of an undifferentiated state

and death of cells. Preferably, the biologically active substance can control the location of cell adhesion to the substrate 1, as well as a cellular function selected from proliferation (including promotion and suppression), differentiation (including promotion and suppression), survival, maintenance of an undifferentiated state, and cell death (apoptosis). Examples of such substance include an extracellular matrix protein, an antibody having a specific binding ability to the cell surface, a cytokine, and a chemical substance that affects cell proliferation or differentiation when bound to or taken into the cells. Examples of the extracellular matrix protein include collagen, fibronectin and laminin. Cytokine includes a cell growth factor and a hormone, and the cell growth factor includes a nerve growth factor (NGF), an epithelium growth factor (EGF), a basic fibroblast growth factor (bFGF), an osteogenesis factor (BMP-2), an insulin-like growth factor (IGF-I), and a tumor necrosis factor (TNF). Examples of hormone include insulin and adrenalin. In one holding area, one or more biologically active substances may be held to the substrate 1. For example, by holding two or more biologically active substances of different functions within the same holding areas, there can be achieved a further advanced control of at least one of

adhesion, proliferation, differentiation, survival, maintenance of an undifferentiated state, and death of cells. The biologically active substance absorbed by a binder such as PVA (polyvinyl alcohol) in a liquid state can be held at a desired position of the substrate 1. Alternatively, a solution of a biologically active substance in an amount as small as several microliters or less can be applied and dried to hold it at a desired position, e.g., on a base 11 and/or a wall 14 and/or a stage 15. Also the held biologically active substance on the substrate 1 may be different in separate holding areas depending on the purpose of cell culture. Also when plural biologically active substances are fixed in predetermined positions in a single area of the substrate 1, the combination of the plural biologically active substances may be the same or different between the separate holding areas. Also, even when the combination of the biologically active substances is the same between the separate holding areas, it is useful to vary concentration ratios. Use of such a substrate 1 allows cell culture with control of at least one function selected from adhesion, proliferation, differentiation, maintenance of undifferentiated state, death etc. of cells under various conditions. In consideration of a possibility that plural biologically active

substances affect the cell culture cooperatively, information of the environment having a largest influence on the cell culture can be obtained by varying the combination of the biologically active substances or concentration ratio thereof in the holding areas.

One or more stages 15 may be provided in the cell culture substrate 1 of the invention. Plural stage 15 at different distances from the base 11 is effective to study the influence of timing of supply of the biologically active substance. By providing holding areas on the base 11, or on the stage 15 and/or on the wall 14 with varying heights from the base 11, and by gradually increasing the amount of the culture medium during cell culture, it is possible to control feeding of plural biologically active substances with time.

[0013]

A cell employable in the present invention may be any procaryotic or eucaryotic cells. For example, there can be employed a bacteria cell, an yeast cell, a neuron, a fibroblast, a smooth muscle cell, a skeletal muscle cell, a gliocyte, an embryonic stem cell, a hematopoietic stem cell, a mast cell, a fat cell, a protozoa cell, a nerve stem cell, an entire of immune cells such as T cell and B cell including transformed or non-transformed cells, or a cluster

thereof.

The base 11 and/or wall 14 and/or stage 15 of the substrate of the invention can be made of any material of any shape, as long as the biologically active substance can be held in a stable manner. More specifically, a glass plate, a plastic plate, a plastic sheet, a polymer film or paper can be employed advantageously. Also the substrate 1 may be transparent, opaque, or colored. Also in order to hold a biologically active substance on the base 11 and/or wall 14 and/or stage 15 or to improve stability of the biologically active substance on the base 11 and/or wall 14 and/or stage 15, the base 11 and/or wall 14 and/or stage 15 or a part thereof may be subjected to a treatment with a chemical substance or a radiation. In case the stage 15 is absent, the wall 14 is preferably inclined in order to provide a holding area. The angle of inclination is preferably about 25 to 65°, since an angle close to vertical makes adjustment of the height of the holding area difficult and a shallower angle reduces the density of wells in the preparation of the cell culture substrate. For forming wells, upright or inclined wall structures in the cell culture substrate, there can be employed, for example, an injection molding, a liquid molding, an adhesion of a chip by thermal fusion or with an adhesive, or a press molding in a

metal mold.

[0014]

Also the base 11 and/or wall 14 and/or stage 15 may be formed in a concave portion (recess or well) formed on the substrate surface. Such configuration can facilitate positioning of a liquid droplet, provided by liquid discharge means onto a predetermined position on the substrate. Further in a well, an area holding a biologically active substance or a group of areas each holding a biologically active substance may be provided, whereby cell culture can be carried out for the respective areas or groups using different culture liquids.

[0015]

A method for producing the cell culture substrate 1 of the above-described configuration will be explained with reference to Fig. 1. First, a base 11 may be subjected to a pretreatment as described above, if necessary. More specifically, the base 11 is washed to eliminate undesired substances and may be subjected to various chemical or physical treatments such as radiation including UV irradiation, and corona discharge. Also it is possible, if necessary, to apply a polymer material or a silane coupling agent on the base 11 and/or wall 14 and/or stage 15 or a part thereof.

[0016]

A biologically active substance is positioned on such base 11 and/or wall 14 and/or stage 15. Liquid discharge means 13, for example, is employed for positioning. The liquid discharge means is capable of discharging a liquid droplet of a volume of 100 nl or less per drop, more specifically about 1 pl to 1 nl, such as a micropipette, a microdispenser, or a discharge apparatus of ink jet method. A discharge apparatus of ink jet method can be employed particularly advantageously because it is inexpensive and can discharge a minute liquid droplet at a controlled position. Furthermore, among the ink jet methods, a thermal ink jet method and a piezo ink jet method can be employed advantageously. A discharge apparatus of the thermal ink jet method, being easy in preparation of fine discharge ports, can discharge a liquid containing a biologically active substance at predetermined positions at a high density, whereby the biologically active substance can be positioned highly precisely on the base 11. Also a discharge apparatus of the piezo ink jet method, in which a discharge energy is generated by displacement of a piezoelectric element, can discharge a liquid 12 containing a biologically active substance without giving a thermal stress thereto.

[0017]

The biologically active substance is dissolved or dispersed in an appropriate solvent for discharge. Any solvent (dispersion medium) may be employed as long as it can stably dissolve or disperse the biologically active substance, but water is employed advantageously. Water is present in 30 mass% or higher, preferably 50 mass% or higher. Preferably, water is ion-exchanged water (deionized water) or various buffers for stably dissolving the biologically active substance. Also, if necessary, a water-soluble solvent may be employed. The amount of each water-soluble solvent to be added is 50 mass% or less, preferably 30 mass% or less. Any water-soluble solvent may be employed as long as it is soluble in water, and examples include an alkyl alcohol with 1 to 4 carbon atoms such as methyl alcohol, ethyl alcohol, n-propyl alcohol, isopropyl alcohol, n-butyl alcohol, sec-butyl alcohol, or t-butyl alcohol; an amide such as dimethylformamide or dimethylacetamide; a ketone or a ketoalcohol such as acetone or diacetone alcohol; an ether such as tetrahydrofuran or dioxane; an polyalkylene glycol such as polyethylene glycol, or polypropylene glycol; an alkylene glycol in which an alkylene group has 2 - 6 carbon atoms such as ethylene glycol, propylene glycol, butylene glycol, triethylene glycol, 1,2,6-hexanetriol, thiodiglycol, hexylene glycol or

diethylene glycol; glycerin; a lower alkyl ether of a polyhydric alcohol such as ethylene glycol monomethyl ether, ethylene glycol monoethyl ether, ethylene glycol monobutyl ether, diethylene glycol monomethyl ether, diethylene glycol monoethyl ether, diethylene glycol monobutyl ether, triethylene glycol monomethyl ether, triethylene glycol monoethyl ether, or triethylene glycol monobutyl ether; N-methyl-2-pyrrolidone, 2-pyrrolidone and 1,3-dimethyl-2-imidazoline. These solvents may be suitably selected in one or more kinds. Among these water-soluble organic solvents, there is preferred a polyhydric alcohol such as diethylene glycol, or a lower alkyl ether such as triethylene glycol monomethyl ether. In case of thermal ink jet method, addition of an alcohol such as ethanol or isopropyl alcohol or a lower alkyl ether of a polyhydric alcohol is advantageous for more stable bubble formation on a thin film resistor in a discharge port of the ink jet head, for providing the biologically active substance.

[0018]

Also a liquid 12 of the invention containing at least the biologically active substance may contain at least a hydrophilic resin. The hydrophilic resin can be, for example, a natural polymer such as a ligninsulfonic acid salt and shellac, a polyacrylic acid salt, a styrene-acrylic acid copolymer salt, or

a styrene-acrylic acid-acrylate ester copolymer salt, an anionic polymer such as a styrene-maleic acid copolymer salt, a styrene-maleic acid-acrylate alkyl ester copolymer salt, a styrene-maleic acid half ester copolymer salt, a styrene-methacrylic acid copolymer salt, a vinylnaphthalene-acrylic acid copolymer salt, a vinylnaphthalene-maleic acid copolymer salt, a β-naphthalenesulfonic acid-formaline condensate salt, or polyphosphoric acid, polyvinyl alcohol, methylolated melamine, polyvinylpyrrolidone, or a cellulose derivative such as methyl cellulose, hydroxymethyl cellulose or carboxymethyl cellulose. In the present invention such resins may be employed singly or in a mixture of two or more kinds. Also there are many other examples, for example a natural resin such as albumin, gelatin, casein, starch, cationized starch, gum Arabic, and sodium alginate. Naturally the present invention is not limited to such examples. By suitably selecting the configuration of a holding layer, it is possible to attain a controlled release of the biologically active substance from the holding layer into a culture liquid. Such controlled release may also be achieved by adding a material capable of realizing a controlled release (for example a water-soluble styrene-acrylic resin) to the liquid containing the biologically active substance. An

amount of the substance capable of realizing the controlled release to the liquid containing the biologically active substance is 10 mass% or less, preferably 5 mass% or less. The aforementioned hydrophilic resin is added within a range of 10 mass% or less per kind, preferably 5 mass% or less.

[0019]

Also the liquid 12 of the invention containing at least the biologically active substance may further include, if necessary for obtaining desired physical properties, a surfactant, a anti-foaming agent, an antiseptic, an inorganic salt, an organic salt and the like. As to the surfactant, any surfactant not detrimentally influencing the biologically active substance for example on a storage stability may be employed, for example an anionic surfactant such as a fatty acid salt, a higher alcohol sulfate ester salt, a liquid fatty oil sulfate ester salt, or an alkylarylsulfonic acid salt, or a nonionic surfactant such as an polyoxyethylene alkyl ether, a polyoxyethylene alkyl ester, a polyoxyethyelensorbitan alkyl ester, acetylene alcohol or acetylene glycol, and these may be employed singly or in a mixture of two or more kinds. Simultaneously with or after the providing of the biologically active substance by the minute droplet discharge means on a desired position of the base 11,

the biologically active substance is held on the base 11 and/or a wall 14 and/or a stage 15. For holding the biologically active substance on the base 11 and/or a wall 14 and/or a stage 15, a treatment may be applied in advance to the biologically active substance or to the base 11 and/or a wall 14 and/or a stage 15. For example, a holding area can be formed by applying an aqueous solution of a water-soluble polymer such as gum Arabic, agar, gelatin, starch, tragacanth, crystalline cellulose, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxypropyl methyl cellulose phthalate, cellulose acetate phthalate, polyvinylpyrrolidone, macrogol, polyvinyl alcohol or methacrylic acid copolymer, or a dispersion of organic or inorganic particles employing such polymer as a binder. The cell culture substrate 1 can be prepared as explained above.

[0020]

In the following, there will be explained a cell culture method utilizing the cell culture substrate 1. By culturing cells under such conditions that at least part of the holding areas in the culture substrate 1 comes in contact with the culture liquid at a predetermined point of culture, it is possible to control at least one of adhesion, proliferation, differentiation, survival, maintenance

of an undifferentiated state and death of cells. The cells are not particularly restricted, but are preferably those of which any of the aforementioned functions may be affected by the held substance. Prior to the cell culture, the cell culture substrate 1 may be sterilized according to necessity, for example, by an ultraviolet irradiation. This operation will prevent contamination of the culture with undesired microorganisms. The cell culture may be conducted by immersing the cell culture substrate 1 in the culture liquid. It is possible to carry out cell culture with controlling at least one of the cell properties mentioned above as long as at least part of the holding areas comes into contact with the culture liquid at a certain point of cell culture. Also it is possible to replace the culture liquid or irradiate with light or radiation during or after cell culture on the substrate 1. In this manner it is possible to vary the proliferation or differentiation of the cells or to vary an adhesive property to the substrate. The cell culture may also be carried out by contacting the biologically active substance with a flow of a culture liquid, namely under a circulation of the culture liquid. Also the cultured cells may be removed from the substrate during or after the cell culture on the substrate 1. In this manner the substrate after the removal of the

cultured cells can be used again, and the removed cultured cells may be utilized as an artificial tissue or a part thereof.

[0021]

Such re-use of the substrate is one of the advantages obtained by immobilizing the biologically active substance to the substrate, whereby the cells are unable to incorporate such an immobilized substance into the metabolic system. Also by coating the substrate in advance with a temperature-responsive polymer such as poly(N-isopropylacrylamide) and culturing cells thereon, it is possible to remove the cultured cells by reducing the temperature to about 30°C, causing a change in the hydrophilicity of the polymer surface. In this manner the cells can be utilized, for example, in a live tissue.

[0022]

For a quantitative determination of a substance synthesized in the cells or a substance incorporated therein, there may be employed a method of measuring an amount of a radiation emitted from a radioactive compound, a method of measuring an amount of fluorescence emitted from a substance labeled with a fluorescent substance, a method of measuring an amount of light emitted from a light-emitting substance, or a method of measuring an absorbance of

a dye.

[0023]

In the method of measuring an amount of a radiation emitted from a radioactive compound, a method employing a compound in which an element abundantly present in a live tissue such as hydrogen, carbon, nitrogen, phosphor or sulfur is replaced by a radioactive isotope, and measuring an amount of radiation emitted from such a compound has a very high sensitivity, and also permits observation of phenomena as in a live body, since such compound is same in chemical properties as in an ordinary compound.

[0024]

Also a method of labeling with a fluorescent substance is relatively simple and gives little influence on the metabolism of the cells by employing a fluorescent substance of a low molecular weight. Also in a quantitative determination of a substance produced by the cells by a determination method utilizing an antigen-antibody reaction, an evaluation by a fluorescent measurement is effective since antibodies labeled with a fluorescent substance are available in various kinds and provide a high measuring sensitivity.

[0025]

Also the method of measuring an amount of

luminescence emitted from a luminescent substance allows to recognize even a small change, since the luminescence can be measured with a high sensitivity. In case a gene is known that is expressed with adhesion, proliferation, differentiation or substance production induced by the screened substance, it is possible to introduce a luciferase gene or the like in the vicinity of such a gene and an amount of luciferase produced by the gene expression is measured from the intensity of luminescence generated on addition of ATP and luciferin. In this manner it is possible to evaluate the influence of the screened substances from the luminescence intensity.

[0026]

In a method of measuring the absorbance of a dye, it is possible to amplify the absorbance by employing an enzyme reaction etc. in combination, thereby enabling a quantitative determination of a substance of a very small amount.

[Examples]

[0027]

In the following, the present invention will be clarified further by examples thereof. In the following description, "%" is based on mass unless specified otherwise.

[0028]

Example 1

The entire surface of a cell culture substrate 1 made of polystyrene was coated with a solution of poly-L-lysine and a dextran activated using tresyl chloride, as an immobilizing crosslinking material.

[0029]

An ink jet cartridge for a thermal ink jet printer, BJF930 (manufactured by Canon Inc.), was sufficiently washed with a 70% aqueous solution of ethanol. IGF-I dissolved in a 10 mM solution of acetic acid was diluted with a 50% aqueous ethanol solution to a concentration of 50 μm/ml. The obtained IGF-I solution was filled in the cartridge of the ink jet printer, then discharged and provided on a base 11. The discharge pattern was controlled by a personal computer connected to the printer, and each droplet had a size of about 4 picoliters. The IGF-I solution was discharged onto the base 11 coated with the active dextran, and the substrate was left to stand in a humidified incubator for 12 hours at 4°C. In this manner there was prepared an IGF-I-immobilizing area. In a similar manner, bFGF, bFGF and IGF-I were immobilized with various combinations and different concentrations according to the areas on the base 11. After the immobilization of the biologically active substances, the unreacted active dextran was blocked with a 1% gelatin solution.

[0030]

On the thus prepared substrate, a murine skeletal muscle cell strain C2C12 was cultured. As a culture liquid, DMEM (Delbucco's modified eagle's minimum essential medium) containing FBS (fetal bovine serum) by 2% was used. At first the culture was conducted for 72 hours at 37°C, in humidified air (95 - 100 %RH) containing CO_2 by 5%, in a state where the level of the culture liquid was lower than the upper surface of the stage 15. Then the cells were treated with 10% formalin for 15 minutes and then with methanol for 15 minutes. Cells were stained with a fluorescent dye TOTO-3 for DNA and with a primary antibody MF20 and a secondary antibody IRDye800 for muscle differentiation, and fluorescent intensities were measured. Fluorescent intensity of TOTO-3 at 700 nm was employed as an index for the cell proliferation on the substrate, and relative fluorescent intensity of IRDye800 as an index for the muscle differentiation.

[0031]

The cell proliferation was accelerated in the areas containing bFGF only depending on the concentration, while the muscle differentiation was accelerated in the areas containing IGF-I only depending on the concentration. However, the muscle differentiation was suppressed in the areas containing both factors, indicating that the function

of IGF-I was suppressed by bFGF. In this manner it was shown that cell differentiation depends on the kinds and combination of the growth factors, even with two growth factors.

[0032]

Example 2

A substrate was prepared employing three growth factors with different combinations and concentrations on the base 11. bFGF, IGF-I and BMP-2 were immobilized on the base 11 in a similar manner as in Example 1. Then the same culture liquid as in Example 1 was added to immerse the stage 15, thereby re-dissolving all the growth factors.

[0033]

Then C2C12 cells were cultured on this substrate for 96 hours in the same manner as in Example 1, and relative activity of creatine kinase (CK) as an index for muscle differentiation, and a relative activity of alkali phosphatase (ALP) as an index for bone differentiation were measured.

[0034]

Results similar to those in Example 1 were obtained in the areas where BMP-2 was not present. With increased concentrations of BMP-2, the relative activity of CK generally decreased and the relative activity of ALP increased. On the other hand, in areas of high BMP-2 concentrations, the relative

activity of ALP decreased with bFGF or IGF-I. It was thus clarified that the muscle differentiation was suppressed while the bone differentiation was accelerated by BMP-2, but the function of BMP-2 was suppressed by IGF-I or bFGF.

[0035]

Next, a culture substrate was prepared holding BMP-2 alone on the stage 15. C2C12 cells were cultured on the substrate in the same manner as above except that BMP-2 did not come into contact with the culture liquid for the first 24 hours. After 24 hours, fresh culture liquid was so added that the stage 15 was immersed, thereby re-dissolving BMP-2. Thereafter the culture was conducted for further 72 hours, and bone differentiation and muscle differentiation on the substrate were measured.

[0036]

As a result, the addition of BMP-2 after 24 hours from the start of culture did not accelerate bone differentiation nor suppress the muscle differentiation, indicating that BMP-2 had to be added within 24 hours from the start of the culture, in order to accelerate bone differentiation. In this manner different states of differentiation could be observed by varying the kinds of the growth factors in combination and the acting time thereof.

[0037]

Example 3

The following process was employed for investigating influence of timing and concentration of a biologically active substance on cells.

[0038]

IGF-I was employed as the biologically active substance. A solution containing glycerin by 5% and IGF-I at 20 μg/ml was prepared. An ink cartridge was washed with 70% ethanol, and filled with the solution containing the biologically active substance. As in Example 1, the base 11 was subjected to a treatment with active dextran.

[0039]

As shown in Fig. 3, IGF-I was discharged by an ink jet printer on the base 11 and the wall 14. IGF-I was immobilized on the wall. After the fixation of IGF-Ion the base 11, the unreacted active dextran was blocked with a gelatin solution. Murine skeletal muscle cell strain C2C12 suspended in DMEM containing 2% FBS was added to a 24 well transparent microplate, thereby dissolving IGF-I on the base 11, and cultured for 96 hours in total at 37°C in wet air containing CO_2 by 5% with addition of the culture liquid at predetermined times after the start of the culture to dissolve IGF-I on the wall 14,

[0040]

after the start of the culture, the culture

liquid was added as follows to dissolve IGF-I:

(1) no addition;

(2) addition after 36 hours; or

(3) additions after 36 hours and 72 hours.

The cells after the culture were treated for 10 minutes with 10% formalin, and stained for the enzyme activity of creatine kinase (CK) as an index of muscle differentiation by IGF-I.

[0041]

As a result, the degree of muscle differentiation varied by the total amount of IGF-I. In the above-mentioned conditions, the stained portion by CK increased in the order of (1) < (2) < (3). This fact indicates that the level of acceleration of muscle differentiation depends on the amount of IGF-I present in the culture liquid throughout the culture period. Thus influence of the action period of a biologically active substance can be studied using a microsubstrate of the present invention that allows addition of the biologically active substance in a stepwise manner.

[0042]

Example 4

The following process was employed for investigating influence of the timing of addition and concentrations of the biologically active substance on cells.

[0043]

IGF-I and bFGF were employed as the biologically active substances. Solutions were prepared to contain 5% glycerin and 20 μg/ml of IGF-I or bFGF. Ink cartridges were washed with 70% ethanol, and filled with the solutions each containing the biologically active substance, respectively. As in Example 1, the base 11 was subjected to a treatment with active dextran.

[0044]

As shown in Fig. 3, IGF-I was immobilized on the base 11, and bFGF was held on the wall 14 by using an ink jet printer to form 16 combinations of concentrations of IGF-I and bFGF in 80 wells (four concentrations for each growth factor, and five wells for each combination). The murine skeletal muscle cell strain C2C12 suspended in DMEM containing FBS by 2% was added to the base 11. Fresh culture liquid was added at predetermined time lapses from the start of the culture to dissolve bFGF on the wall 14. Cells were cultured for 96 hours at 37°C and in humidified air containing CO_2 by 5%. Addition of fresh culture liquid was conducted as follows:

(1) Immediately after the start of culture;

(2) After 12 hours from the start of culture;

(3) After 24 hours from the start of culture;

(4) After 48 hours from the start of culture;

(5) After 72 hours from the start of culture.

The cells after the culture were treated for 10 minutes with 10% formalin, and subjected to enzyme activity staining for creatine kinase (CK) as an index for muscle differentiation with IGF-I.

[0045]

As a result, the muscle differentiation was affected by the timing of action of bFGF. Many stained sites were observed where bFGF was not disposed on the wall 14 under conditions (4) and (5), while substantially no stained site was observed under conditions (1), (2) or (3). Also the number of stained sites decreased as the bFGF concentration increased. This fact indicates that, the muscle cell differentiation is significantly suppressed when bFGF is added within 24 hours from the start of the culture. Thus influence of the action period of a biologically active substance can be studied using a microsubstrate of the present invention that allows addition of the biologically active substance in a stepwise manner.

[0046]

Example 5

This Example was carried out to assess the change in mutagenicity of a mutagen, where Salmonella strain TA98 was cultured on a substrate with various combinations of the mutagen and other substances to

quantitatively determine the reverse mutation of strain TA98.

In this Example, three mutagens, i.e., sodium azide, aflatoxin B1 and acetohexamide were temporarily immobilized on a substrate covered with agar containing glucose, where these mutagens were immobilized in specified regions as shown in FIG. 5 with different concentrations. In FIG. 5, ◎ represents a region where no mutagen was temporarily immobilized; open circle and filled circle represent regions where sodium azide was temporarily immobilized; vertically striped circles represent regions where aflatoxin was temporarily immobilized; and dotted circles represent regions where acetohexamide was temporarily immobilized. Region where a mutagen was applied in a higher concentration is shown by a denser circle, that is, a filled circle, a densely striped circle or a densely dotted circle. Each mutagen was immobilized alone and in combination with other mutagens.

Sodium azide, aflatoxin B1 and acetohexamide were separately dissolved in a sodium-sodium phosphate buffer (pH 7.0) and each solution was filled in an ink cartridge previously washed with 70% ethanol. By using an ink jet printer, each mutagen was applied on the above described substrate and temporarily immobilized.

According to EISEI SHIKENHOU (Japanese Standard methods of analysis for hygienic chemists), 0.1 ml of cell suspension of Salmonella strain TA98 grown in a liquid growth medium was mixed well with 2 ml of an agar solution containing L-histidine and D-biotin, and the mixture was spread on the above-described substrate. After the added agar was solidified on the substrate at room temperature, the substrate was incubated at 37°C for 48 hours and the colonies grown due to reverse mutation were counted.

As a result, in the regions where mutagens were separately immobilized, the number of colonies increased depending on the mutagen concentrations so that the mutagenicity of each mutagen was confirmed. However, when both sodium azide and aflatoxin B1 were present, the bacteria were killed off. This shows cytotoxity becomes stronger than mutagenicity in this combination.

On the other hand, when sodium azide and acetohexamide were both present, the colony number remarkably increased in comparison with the colonies formed with respective mutagens, showing synergic action in mutagenicity. When aflatoxin B1 and acetohexamide were added together, no synergic action was observed. When three mutagens were combined, cells were killed off showing stronger cytotoxity.

As shown above, use of present substrate

enables evaluation of change in mutagenicity or cytotoxity when plural substances are combined.

[0047]

Example 6

This Example was carried out to assess the change in mutagenicity of a mutagen when the mutagen was combined with other substances with time lag, by quantitatively determining reverse mutation rates of Salmonella strain TA98. In this Example, mutagens, i.e., sodium azide and aflatoxin B1 were immobilized in different concentrations in specified regions on a substrate covered with agar containing glucose as shown in FIG. 6. In FIG. 6, a double circle represents a region where no mutagen was immobilized; open circle and filled circle represent regions where sodium azide was immobilized; vertically striped circles represent regions where aflatoxin was immobilized. Region where a mutagen was applied in a higher concentration is shown by a denser circle, that is, a filled circle or a densely striped circle. Each mutagen was immobilized alone and in combination with the other mutagens.

[0048]

Sodium azide and aflatoxin B1 were separately dissolved in a sodium-sodium phosphate buffer (pH 7.0) and each solution was filled in an ink cartridge previously washed with 70% ethanol. By using an ink

jet printer, each mutagen was applied on the above described substrate and immobilized.

[0049]

0.1 ml of cell suspension of Salmonella strain TA98 grown in a liquid culture medium was added to respective 0.5 ml of a liquid culture medium containing acetohexamide in various concentrations and cultured at 37°C for 20 minutes with shaking. Then each culture was mixed well with 2 ml of an agar solution containing L-histidine and D-biotin, and each mixture was put on the substrate. After the added agar was solidified on the substrate at room temperature, the substrate was incubated at 37°C for 48 hours and the colonies grown due to reverse mutation were counted. The above process was repeated except that the combination of mutagens to be immobilized and one to be added later were changed among three mutagens. As a result, in the regions where mutagens were separately immobilized, the number of colonies increased depending on the mutagen concentration to confirm mutagenicity. However, when the cells were precultured with acetohexamide and then brought into contact with sodium azide and aflatoxin B1, cells were not killed off, that is, cytotoxity became weaker than the cytotoxity shown by the three mutagens added at the same time. It was also shown that mutagenicity was changed on the whole

by the time lag addition. As shown above, use of the present substrate enables to evaluate changes in mutagenicity and cytotoxity caused by combination of plural substances and time lag addition thereof.

[Industrial Applicability]

[0050]

As explained in the foregoing, the present invention enables to analyze growth factors in a complex multiple system simply and within a short time, and therefore has a wide application in the fields of bioscience, biochemical science and regeneration therapy.

[Brief Description of the Drawings]

[0051]

[Figure 1] A diagram illustrating an example of preparation of a cell culture substrate of the present invention.

[Figure 2] A plan view of an example of a cell culture substrate of the present invention.

[Figure 3] A cross-sectional view of an example of a cell culture substrate of the present invention.

[Figure 4] A cross-sectional view of an example of a cell culture substrate of the present invention.

[Figure 5] A plan view illustrating an example of a cell culture substrate of the present invention.

[Figure 6] A diagram illustrating an example of a mutagenic substance screening method.

[Description of Reference Numerals or Symbols]

[0052]

1: cell culture substrate

2: growth factor A

3: growth factor B

4: growth factor C

5: growth factor D

6: growth factor E

11: base

12: liquid containing biologically active substance

13: discharge means

14: wall

15: stage

16: inclined wall

17, 17': sodium azide temporarily immobilized area

18, 18': aflatoxin B1 temporarily immobilized area

19, 19': acetohexamide temporarily immobilized area

20, 20': fungus suspension pre-cultured with acetohexamide solution

21, 21': sodium azide immobilized area

22, 22': aflatoxin B1 immobilized area

[Name of the Document] Abstract

[Abstract]

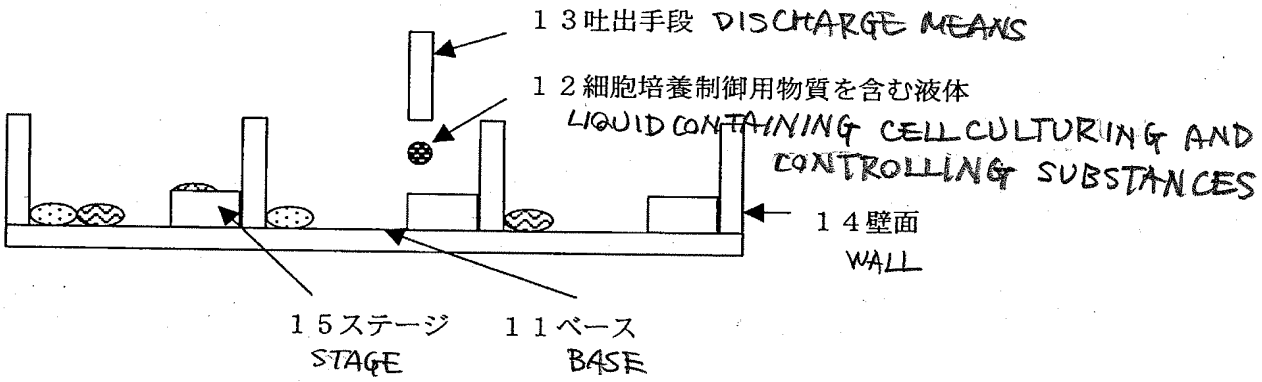
[Subject] To provide a cell screening substrate which can be formed by simple steps and a method of producing the cell screening substrate.

[Solving Means] A holding area serving as a source for supplying a biologically active substance having a biological activity to a cell and an area for immobilizing the biologically active substance are provided in each of a plurality of culturing areas on a substrate. The cell culturing substrate includes a holding area from which at least one biologically active substance can be released and is configured such that a plurality of areas which are different from one another in combination of biologically active substances and/or a plurality of areas which are different from one another in density of biologically active substances are present on a plurality of parts which are different from one another in elevation on the cell culturing substrate.

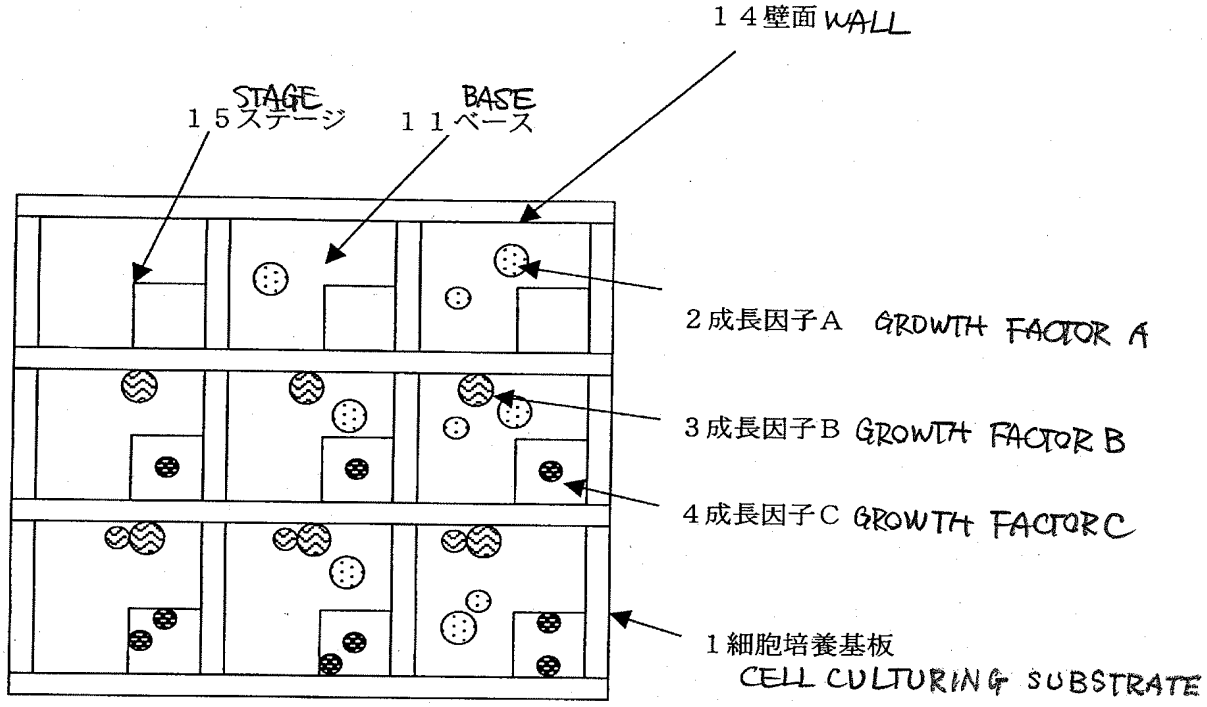
[Elected Drawing] Figure 1

【書類名】図面 [Name of the Document] Drawings

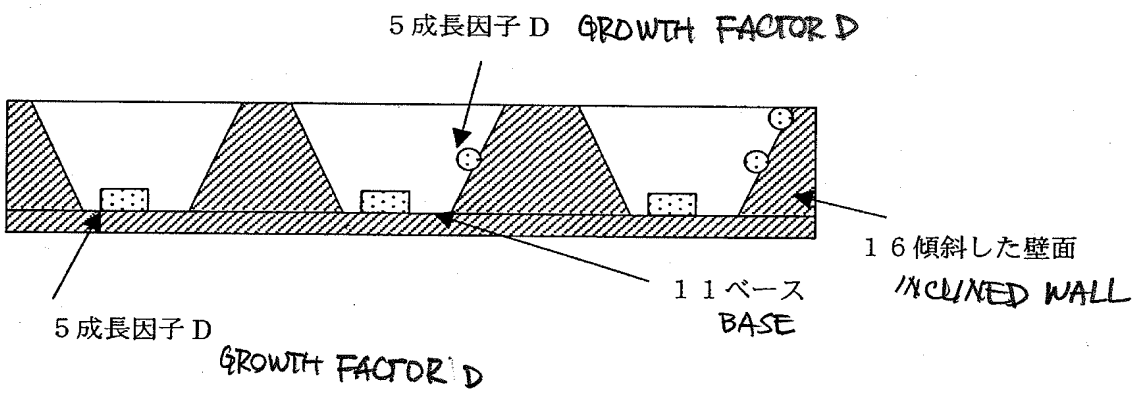
【図１】 [Fig. 1]



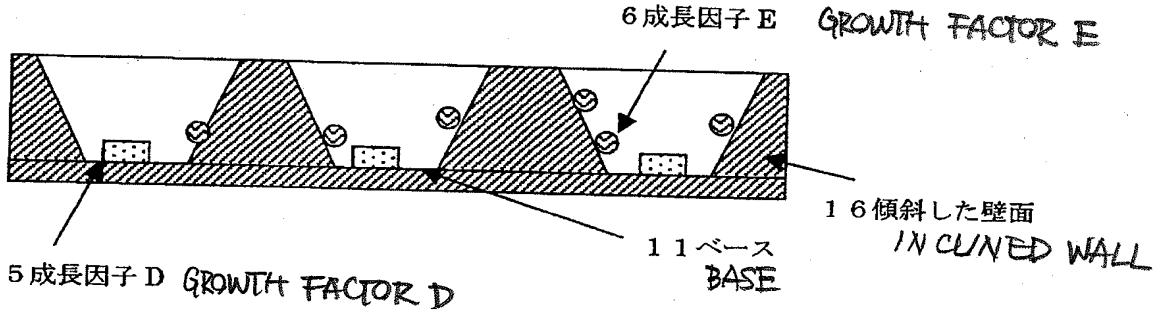
【図２】 [Fig. 2]



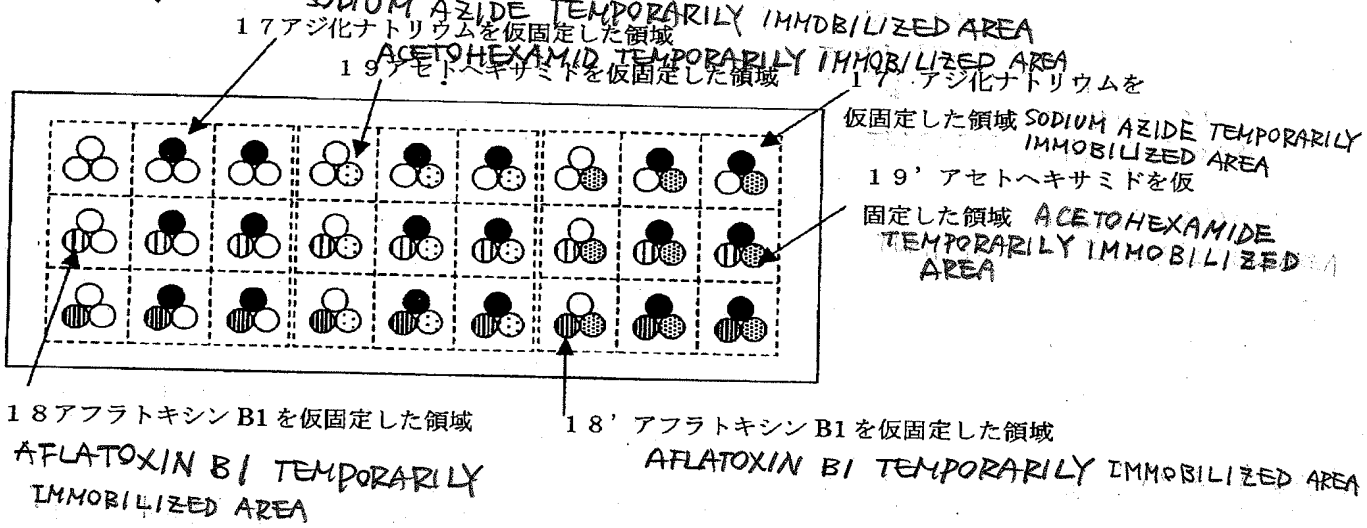
【図３】 [Fig. 3]



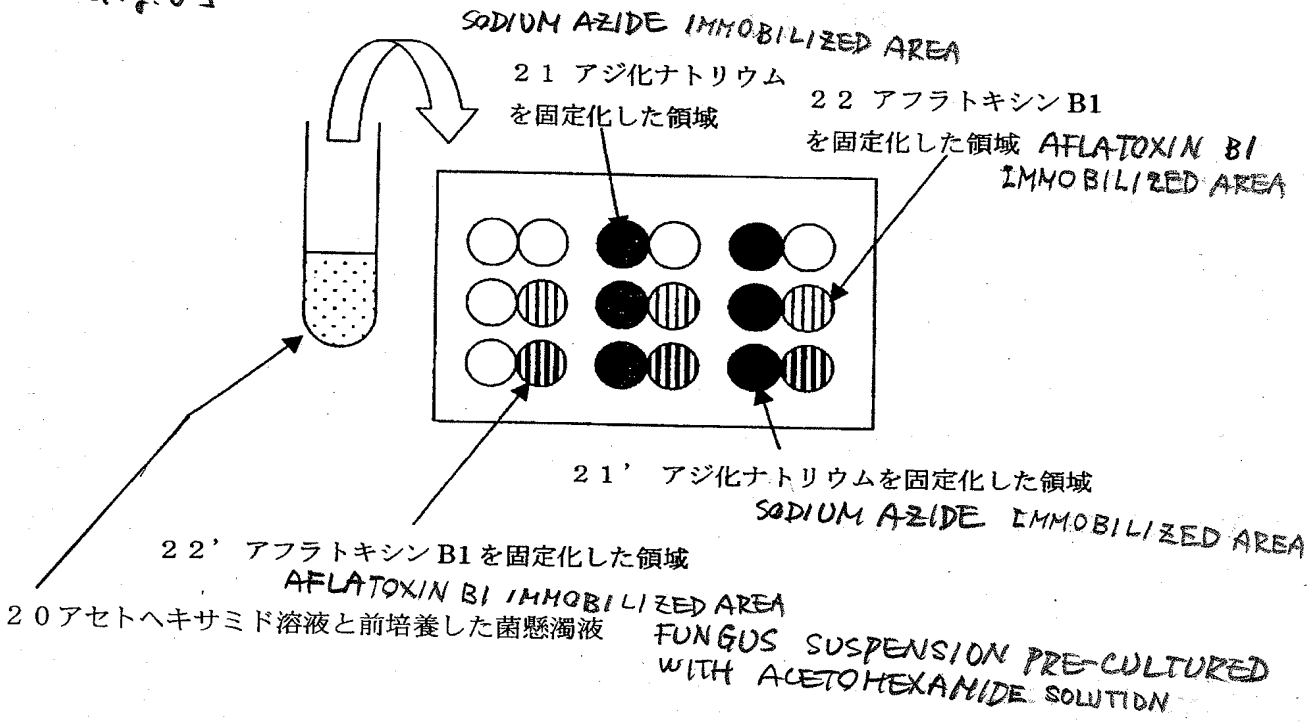
【図４】 [Fig. 4]



【図５】 [Fig. 5]



【図６】 [Fig. 6]


SODIUM AZIDE IMMOBILIZED AREA
２１ アジ化ナトリウム
を固定化した領域
２２ アフラトキシンB1
を固定化した領域 AFLATOXIN B1
IMMOBILIZED AREA
２１’ アジ化ナトリウムを固定化した領域
SODIUM AZIDE IMMOBILIZED AREA
２２’ アフラトキシンB1を固定化した領域
AFLATOXIN B1 IMMOBILIZED AREA
２０アセトヘキサミド溶液と前培養した菌懸濁液
FUNGUS SUSPENSION PRE-CULTURED
WITH ACETOHEXAMIDE SOLUTION

2003-418560

Applicant's Information

Identification No. [000001007]

1. Date of Change: August 30, 1990

(Reason of Change) New Registration

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